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Peroxisome degradation in *Saccharomyces cerevisiae* is dependent on machinery of macroautophagy and the Cvt pathway

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SUMMARY

Organelle biogenesis and turnover are necessary to maintain biochemical processes that are appropriate to the needs of the eukaryotic cell. Specific degradation of organelles in response to changing environmental cues is one aspect of achieving proper metabolic function. For example, the yeast *Saccharomyces cerevisiae* adjusts the level of peroxisomes in response to differing nutritional sources. When cells are grown on oleic acid as the sole carbon source, peroxisome biogenesis is induced. Conversely, a subsequent shift to glucose-rich or nitrogen-limiting conditions results in peroxisome degradation. The degradation process, pexophagy, requires the activity of vacuolar hydrolases. In addition, peroxisome degradation is specific. Analyses of cellular marker proteins indicate that peroxisome degradation under these conditions occurs

more rapidly and to a greater extent than mitochondrial, Golgi, or cytosolic protein delivery to the vacuole by the non-selective autophagy pathway. To elucidate the molecular mechanism of selective peroxisome degradation, we examined pexophagy in mutants that are defective in autophagy (*apg*) and the selective targeting of aminopeptidase I to the vacuole by the cytoplasm to vacuole targeting (Cvt) pathway. Inhibition of peroxisome degradation in *cvt* and *apg* mutants indicates that these pathways overlap and that peroxisomes are delivered to the vacuole by a mechanism that utilizes protein components of the Cvt/autophagy pathways.

Key words: Aminopeptidase I, Autophagy, Cvt pathway, Peroxisome degradation, Pexophagy, Vacuole, Yeast

INTRODUCTION

Some organelles, such as the peroxisome, fluctuate in number and size according to the physiological needs of the cell. Sufficient peroxisomes are required to carry out fatty acid β -oxidation when *Saccharomyces cerevisiae* encounters oleic acid as its sole carbon source (Veenhuis, 1987). Many of the proteins involved in peroxisome biogenesis have been identified in both *S. cerevisiae* as well as the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* (van der Klei and Veenhuis, 1997; Waterham and Cregg, 1997; Subramani, 1998; Kunau and Erdmann, 1998). Cells exact an energetic cost to maintain organelles in a functional state. Accordingly, excess organelles are degraded when they are no longer needed.

Most of our current understanding of peroxisome degradation, or pexophagy, comes from experiments in *P. pastoris* and *H. polymorpha* (reviewed by Klionsky, 1997). Electron microscopy studies have shown membrane structures surrounding peroxisomes within the vacuole under conditions which induce pexophagy in these yeasts (Tuttle and Dunn, 1995; Yuan et al., 1997; Titorenko et al., 1995). In *H. polymorpha*, peroxisomes are degraded by macropexophagy (Veenhuis et al., 1983). In this process, a membrane of

unknown origin enwraps peroxisomes in the cytosol. The sequestering membrane is at least double-lamellar so that subsequent fusion with the vacuole releases a membrane-enclosed peroxisome into the vacuole lumen. In *P. pastoris*, degradation occurs by both micro- and macropexophagic mechanisms, depending on whether the carbon source is glucose or ethanol, respectively (Tuttle and Dunn, 1995). In micropexophagy, uptake of peroxisomes occurs at the vacuole surface. In either case, the degradative process appears to be specific, suggesting some type of recognition event. Other organelles such as mitochondria are not degraded under conditions that induce pexophagy. Recently, genetic studies have led to the isolation of mutants defective in peroxisome degradation.

The *H. polymorpha* peroxisome degradation deficient mutants *pdd1* and *pdd2* are defective in the initial sequestration event and in fusion of enwrapped peroxisomes with the vacuole, respectively (Titorenko et al., 1995). The Pdd1 protein is homologous to *S. cerevisiae* Vps34p, a phosphatidylinositol 3-kinase (Kiel et al., 1999). The *S. cerevisiae* Vps34p interacts with a protein kinase, Vps15p (Stack et al., 1995). Recently, a *P. pastoris* Vps15p homolog required for selective peroxisome autophagy was identified (Stasyk et al., 1999). Initially, two mutants were identified in the glucose-induced selective

autophagy pathway of *P. pastoris*, *gsa1* and *gsa2* (Tuttle and Dunn, 1995). The *gsa1* mutant is blocked in sequestration of peroxisomes by micropexophagy. This mutant has a defect in the *PFK1* gene encoding the regulatory subunit of phosphofructokinase. The role of this protein in pexophagy is not clearly defined and appears to be independent of phosphofructokinase activity.

The more recently isolated *gsa7* mutant is blocked in the final stage of sequestration by micropexophagy (Yuan et al., 1999). The *GSA7* gene is homologous to the *S. cerevisiae* *APG7/CVT2* gene (Yuan et al., 1999; Kim et al., 1999; Tanida et al., 1999). Apg7p shares homology with the E1-family of ubiquitin activating enzymes, and functions in a novel non-ubiquitin protein conjugation event that is required for API import through the Cvt pathway and macroautophagy (Mizushima et al., 1998). Additional complementation groups have been characterized for the *pdd* and *gsa* mutants. In addition, a separate screen has identified a set of mutants in *P. pastoris* defective in peroxisome microautophagy, or *pag* (Sakai et al., 1998). Finally, peroxisome degradation deficient mutants have recently been isolated in the non-methylotrophic yeast *Yarrowia lipolytica* (Gunkel et al., 1999). At present, however, none of the genes that complement these additional mutants have been characterized.

It is clear that both micro- and macropexophagy are used for peroxisome degradation in the methylotrophic yeasts. In contrast, it has not been determined which degradative mechanism(s) is used in *S. cerevisiae*. The vacuole-dependent degradation of peroxisomes via a multi-lamellar intermediate that forms during macropexophagy is topologically similar to macroautophagic degradation of bulk cytosol. Macroautophagy has been characterized in *S. cerevisiae* through electron microscopy studies (Takeshige et al., 1992; Baba et al., 1994, 1995). This process involves the formation of a double-membrane vesicle, the autophagosome, that non-specifically sequesters cytosol. Subsequent fusion with the vacuole delivers the contents to the lumen for degradation. Evidence for similar autophagic processes has been seen in mammalian and plant cells (Dunn, 1994; Moriyasu and Ohsumi, 1996; Aubert et al., 1996). The signals that trigger pexophagy, however, such as the addition of glucose to oleic acid-grown cells, are essentially the opposite of the starvation signals that induce autophagy. In addition, autophagy is a non-specific process. Uptake of bulk cytosol by autophagy plateaus at approximately 30%. In contrast, pexophagy results in a nearly complete loss of peroxisomes.

Recent work in our laboratory has revealed overlapping characteristics between autophagy mutants (*apg*, *aut*: Tsukada and Ohsumi, 1993; Thumm et al., 1994) and mutants involved in a biosynthetic mechanism of protein targeting to the vacuole, the cytoplasm to vacuole targeting (Cvt) pathway used for import of the resident hydrolase aminopeptidase I (API) (Harding et al., 1995; Scott et al., 1996). While autophagy is non-selective, the overlap with the Cvt pathway shows that the subcellular machinery utilized for autophagy is also required for a selective targeting process. In light of this, pexophagy may also utilize components from the autophagic pathway, as well as unique components that are involved in both signal transduction and to specifically mark excess peroxisomes for degradation.

It is important to understand how excess peroxisomes are

degraded in the cell because of their significance in cellular metabolism. To gain further insight into the molecular basis for pexophagy, we have undertaken an analysis of peroxisome degradation in *S. cerevisiae*. In this report, we present evidence for a specific uptake of excess peroxisomes into the vacuole for degradation after a shift to glucose rich, nitrogen starvation conditions. Mutants in autophagy and the Cvt pathway have been found to be defective in the degradation process. These data demonstrate an overlap among these three pathways and suggest that many of the proteins required for pexophagy are encoded by the *CVT*, *APG* and *AUT* genes.

MATERIALS AND METHODS

Reagents

Restriction enzymes were from New England BioLabs (Beverly, MA). Expre^{35S} Protein Labeling Mix was from Dupont-NEN Research Products (Boston, MA). Immobilon-P (polyvinylidene fluoride, PVDF) was from Millipore (Bedford, MA). Oxalyticase was from Enzogenetics (Corvallis, OR). Yeast nitrogen base (YNB) was from Difco (Detroit, MI). Pefabloc was from Roche Biochemicals (Indianapolis, IN). Goat anti-rabbit IgG alkaline phosphatase was from Jackson Immuno Research (West Grove, PA). Vistra Kit western blotting reagents were from Amersham (Arlington Heights, IL). Antiserum to phosphoglycerate kinase (PGK) was from Dr Jeremy Thorne (University of California, Berkeley). Antisera to mitochondrial F₁ ATPase subunit β (F₁ β) were from Drs Michael G. Douglas (Sigma Aldrich Corporation) and Michael P. Yaffe (University of California, San Diego). All other reagents were from Sigma Chemical Co. (St Louis, MO).

Strains and plasmids

The strains used in this study are listed in Table 1. Standard methods were used to construct the following strains: strain MHY1 was generated by deletion of the *APG7* gene (Kim et al., 1999) in strain MMY011 (McCammon et al., 1990) through one-step gene disruption. Strain MHY8 was generated by transforming YW5-1B with the *EcoRI/XhoI* fragment of pTS15 bearing *pep4 Δ ::URA3* (Rothman et al., 1986). Strain MHY9 was generated from YW5-1B utilizing the *EcoRI* fragment of the *pho8 Δ ::TRP1* plasmid described by Klionsky and Emr (1989).

Plasmid pMUH8 bearing the Pho8 Δ 60p cytosolic marker was generated from the *NaeI/ScaI* fragment of pCC5 (Campbell et al., 1998) ligated into the same sites of pRS415 (Sikorski and Hieter, 1989).

Growth and labeling conditions

Exponential phase cultures in YPD (1% yeast extract, 2% peptone, 2% glucose) were subcultured to SGD (0.67% YNB without amino acids and brought to pH 5.5 with 50 mM each 2-(*N*-morpholino) ethanesulfonic acid and 3-(*N*-morpholino) propanesulfonic acid, 3% glycerol, 0.1% glucose) at 0.3-0.5 OD₆₀₀ and incubated 12 hours at 30°C. A 10 \times YP solution was then added to a final concentration of 1% yeast extract and 2% peptone. After four hours, cells were harvested, washed in sterile H₂O, and resuspended in YTO (0.67% YNB without amino acids, 0.1% Tween-40, and 0.1% oleic acid). The induction period for peroxisomes was 19 hours on YTO. Nitrogen starvation conditions were in SD-N (0.67% YNB without amino acids or ammonium sulfate, 2% glucose). All media were supplemented with required amino acids. For radiolabeling experiments, cells were cultured in SMD (Scott et al., 1996). After subsequent manipulations as above, cells were resuspended to 20 OD₆₀₀ units/10 ml YTO, labeled with 1100 μ Ci Expre^{35S}/ml and the cells were incubated for 19 hours. Following the labeling period, the cells were harvested

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference
UTL-7A	<i>Mata ura3-52 trp1 leu2-3,112</i>	Erdmann and Kunau, 1994
<i>fox3-2</i>	UTL-7A <i>fox3-2</i>	Erdmann and Kunau, 1994
<i>pex7Δ</i>	UTL-7A <i>pex7Δ::LEU2</i>	Marzioch et al., 1994
MMY011	<i>MATα ade1-2, his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100, Ole+</i>	McCammon et al., 1990
MHY1	MMY011 <i>apg7Δ::LEU2</i>	This study
YW5-1B	<i>Mata ura3-52 trp1 leu2-3,112</i>	Noda et al., 1995
TN122	YW5-1B <i>pho13Δ::LEU2 pho8Δ::pho8Δ60 prb1::TRP1</i>	Noda et al., 1995
NNY20	<i>Mata trp1 ura3 leu2 apg1Δ::LEU2</i>	Y. Ohsumi
MT37-4-3	<i>Mata apg5-1 ura3</i>	Y. Ohsumi
MT87-4-5	<i>Mata apg7-1 ura3</i>	Y. Ohsumi
MT28-4-4	<i>Mata apg8-1 ura3</i>	Y. Ohsumi
MT91-4-2	<i>Mata apg10-1 ura3</i>	Y. Ohsumi
MT54-4-2	<i>Mata apg14-1 ura3</i>	Y. Ohsumi
MHY8	YW5-1B <i>pep4Δ::URA3</i>	This study
MHY9	YW5-1B <i>pho8Δ::TRP1</i>	This study

by centrifugation and the cell pellet was washed in sterile H₂O and resuspended in SD-N containing a chase solution of 0.02/0.01 mM cold methionine/cysteine, respectively.

Immunoblotting

Two synthetic peptides were generated (Multiple Peptide Systems, San Diego, CA) corresponding to amino acid residues 26-41 and 197-212 of the *FOX3* gene product 3-ketoacyl-CoA thiolase. These peptides were conjugated at the N terminus to keyhole limpet hemocyanin, emulsified with Freund's adjuvant and 0.5 mg of each peptide was injected into a New Zealand White male rabbit. Antigen boosts (0.06 mg of each peptide) were given at approximately 4-6 week intervals after the first injection. Samples were resolved on 8 or 10% SDS-PAGE gels, transferred to PVDF membrane, and probed essentially as described previously (Harding et al., 1995). Fox3p antiserum was used in a 1:10,000 dilution for 2 hours at room temperature. Antiserum to PGK was used at 1:300,000 dilution. Sample load for PGK immunoblots was 10% of those for other antibodies. Secondary antibodies were goat anti-rabbit IgG alkaline phosphatase for Vistra Kit detection as directed by the manufacturer. Data from immunoblots and immunoprecipitations were quantified on a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation

Labeled cells (see Growth and Labeling Conditions) were harvested, washed and the cell pellet was resuspended in SD-N chase medium. Cell lysis and immunoprecipitation were done essentially as previously described (Klionsky et al., 1992) using 3 μl of F1β or 10 μl of Kex2p antiserum (Fuller et al., 1989). The antigen-antibody complex bound to Protein A-Sepharose was washed once with IP buffer (Klionsky et al., 1992), twice with IP buffer plus 2 M urea, and once each with 1% β-mercaptoethanol and 0.1% SDS before being eluted in sample buffer containing 5% β-mercaptoethanol. To detect Pho8Δ60p, double immunoprecipitations were performed with antiserum to Pho8p (Klionsky and Emr, 1989).

Electron microscopy

Cells were induced for peroxisomes as described above and chased in SD-N where indicated. 20 OD₆₀₀ units were harvested at each timepoint and washed in 0.1 M sodium cacodylate buffer, pH 7.2. Cells were fixed in a mixture of 2.5% formaldehyde and 0.5% glutaraldehyde in sodium cacodylate buffer, pH 7.2, for 60 minutes at 0°C, then dehydrated in an ethanol series and embedded in Unicryl. Preparation of fixed cells and immunolabeling with Fox3p antiserum for electron microscopy was essentially as described (Marzioch et al., 1994).

Subcellular fractionation

Approximately 60 OD₆₀₀ units were shifted to YPD for 30 minutes

after peroxisome induction to facilitate spheroplasting. 10 μg/OD₆₀₀ unit of oxalyticase was added to SMD, pH 7.4, containing 1 M sorbitol after a 15 minute preincubation in 10 mM Tris sulfate, pH 9.4, 1 mM DTT. Incubation at 30°C for 50 minutes resulted in 80-90% spheroplasting as observed by OD₆₀₀ readings. Cells were harvested at 3,000 rpm for 3 minutes, resuspended to 20 OD₆₀₀/ml in a 1.7 ml tube and harvested again in a microcentrifuge. 1 ml of physiological salts buffer (100 mM KOAc, 50 mM KCl, 0.5 mM MgCl₂, 30 mM K-PIPES, pH 6.8) plus 4 mM PefaBloc and 1 μg/ml Pepstatin A was added and pipetted 15 times at room temperature to osmotically lyse the cells. Lysed cells were centrifuged at 800 rpm for 5 minutes at 4°C to clear unlysed spheroplasts. An aliquot of the resulting supernatant was taken as the total sample and TCA precipitated (10%) on ice 1 hour. The remaining supernatant was spun at 25,000 rpm in a Beckman ultracentrifuge (Fullerton, CA) with a TLA100.4 rotor at 4°C. Samples of the 25,000 rpm supernatant and pellet were resolved by SDS-PAGE and immunoblotted as described.

RESULTS

Fox3p is a marker for peroxisomes

Fox3p (3-ketoacyl-CoA thiolase) is localized to the peroxisome matrix where it acts as a dimer in fatty acid β-oxidation (Glover et al., 1994). We generated antiserum to Fox3p to allow us to monitor the level of this peroxisomal matrix enzyme under various environmental conditions. Wild-type strains produce detectable Fox3p when grown on medium containing oleic acid as the sole carbon source. Because Fox3p is specifically induced under peroxisome proliferating conditions and is targeted to the peroxisome with a rapid rate (Glover et al., 1994), we chose to monitor its levels as an indication of the peroxisome level in the cell. When *Saccharomyces cerevisiae* yeast cells were shifted to YTO medium, a 45kDa band was observed after approximately 2 hours (Fig. 1) and increased throughout the induction period (data not shown). This band was not detectable in a *fox3-2* mutant (Fig. 1; Erdmann and Kunau, 1994) or a strain disrupted at the chromosomal *FOX3* locus (data not shown), indicating that the antibodies are recognizing the authentic Fox3 protein.

Degradation of Fox3p is a specific, vacuole-dependent process

Methylotrophic yeasts including *Pichia pastoris* and *Hansenula polymorpha* degrade excess peroxisomes when cells are shifted from oleic acid or methanol to a preferred carbon source such as glucose or ethanol that fully represses

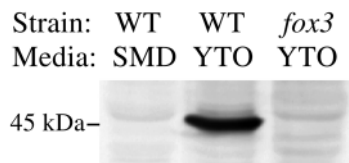


Fig. 1. Antiserum to the peroxisomal protein Fox3p (3-ketoacyl-CoA thiolase) recognizes a specific protein that is induced in wild-type yeast upon shift to oleic acid media. Cells were grown in rich medium (SMD) or under peroxisome-inducing conditions (YTO) as described in Materials and Methods. Yeast cell extracts were resolved on 10% SDS polyacrylamide gels and transferred to PVDF membrane. Immunoblots were probed with antiserum to Fox3p. WT, UTL-7A wild-type strain; *fox3*, mutant *fox3-2* (Erdmann and Kunau, 1994) transcribes a truncated transcript which yields no antigenically detectable Fox3p. A strain disrupted at the chromosomal *FOX3* locus showed essentially identical results as the *fox3-2* strain.

the synthesis of peroxisomal enzymes. We examined the fate of peroxisomal Fox3p in *S. cerevisiae* when that yeast is shifted from peroxisome inducing medium to nitrogen starvation conditions. In order to induce degradation of peroxisomes, we utilized a glucose rich, nitrogen limiting medium (SD-N). The omission of nitrogen limits the rate of growth and avoids the dilution effect encountered upon cell division and partitioning of peroxisomes into daughter cells as seen by the alternate method of adding glucose to cultures. Cells were incubated in YTO medium to induce peroxisomes as described in Materials and Methods, followed by a shift to SD-N medium. Samples were removed over a twenty hour time course and protein extracts were analyzed by immunoblot. Immunoblot analysis of Fox3p revealed it was degraded under these conditions to 50% the level present at *t*₀ in approximately 3.2–5.2 hours (Fig. 2A). In addition, there was a >90% loss of immunologically detectable Fox3p by twenty hours. Under the same conditions, a strain deficient in the vacuolar hydrolases proteinase A (Fig. 2B) or proteinase B (Fig. 3F) was blocked in Fox3p degradation. The observation that loss of Fox3p was dependent on vacuolar enzymes suggests that peroxisomes were degraded in the vacuole. This is in agreement with the data from *P. pastoris* and *H. polymorpha* where pexophagy occurs through engulfment by, or fusion with, the vacuole (Tuttle and Dunn, 1995; Sakai et al., 1998; Titorenko et al., 1995). We also examined loss of Fox3p when cells were shifted from YTO to a glucose-containing rich medium, YPD. Essentially similar results were seen after correcting for loss of signal due to cell doubling (data not shown).

The kinetics of degradation that we observed under nitrogen starvation conditions in *S. cerevisiae* were slower than those reported for the loss of alcohol oxidase in the methylotrophic yeasts (Sakai et al., 1998; Yuan et al., 1997; Titorenko et al., 1995). To determine if the degradative process that we were monitoring was specific, we examined marker proteins for additional organelles and bulk cytosol. A truncated form of alkaline phosphatase lacking its amino-terminal transmembrane domain, Pho8Δ60p, serves as a convenient marker for the non-specific uptake of cytosolic proteins (Noda et al., 1995). Pho8Δ60p can only enter the vacuole through autophagy but retains a propeptide domain that allows a simple method for following vacuolar delivery. We monitored the vacuolar uptake of Pho8Δ60p, F₁β, a subunit of the mitochondrial ATPase, and

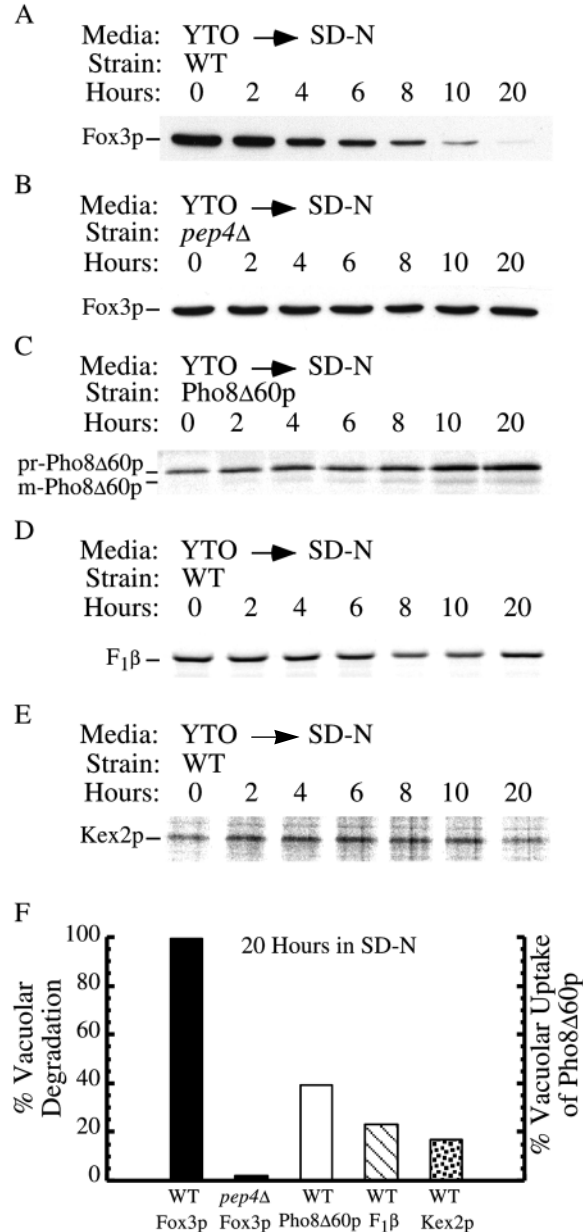


Fig. 2. Degradation of Fox3p is a specific process. Exponential phase cells of strain (A,D,E) WT (YW5-1B), (B) *pep4Δ* (MHY8), (C) Pho8Δ60p (MHY9 transformed with pMUH8) were induced for peroxisomes and shifted to degradation conditions as described in Materials and Methods. Samples were taken at the indicated times after cells were shifted to SD-N, precipitated by the addition of TCA (10%) on ice, and prepared for (A,B) immunoblot or (C-E) immunoprecipitation analysis. Antisera were directed against (A,B) Fox3p, (C) the cytosolic marker Pho8Δ60p, (D) the mitochondrial marker F₁β, or (E) Golgi marker Kex2p. (F) Immunoblots and immunoprecipitation data for time zero and 20 hours were quantified on a PhosphorImager (A,B: solid bar, C: open bar, D: diagonal hatched bar, and E: stippled bar). The percentage degradation was calculated as the ratio of the signal at time 20 hours divided by the signal at time zero (A,B,D,E). The percentage vacuolar uptake of Pho8Δ60p is shown as the level of mature Pho8Δ60p at time 20 hours divided by the total Pho8Δ60p at time zero. Degradation of Fox3p (peroxisomes) occurs more rapidly and to a greater extent than is seen for bulk cytosol or organellar markers, following shift to nitrogen starvation conditions.

Kex2p, a marker for the trans Golgi network under the same conditions used to induce pexophagy (Fig. 2).

Uptake of Pho8Δ60p, F₁β, and Kex2p occurred at a slower rate and to a lesser extent than that of Fox3p. Following a shift to SD-N medium, Pho8Δ60p was taken into the vacuole at a rate much slower than was seen for Fox3p. In addition, the total level of Pho8Δ60p import into the vacuole peaked at approximately 40% in agreement with the previously published characteristics for this protein (Noda et al., 1996; Scott et al., 1996). Furthermore, vacuolar uptake of Pho8Δ60p is essentially undetectable in rich medium (Scott et al., 1996). In contrast, loss of Fox3p was still seen under these conditions (data not shown). Similar to the result with Pho8Δ60p, we only detected a 17–23% loss of the Golgi and mitochondrial marker proteins Kex2p or F₁β, respectively, following the shift to SD-N (Fig. 2D,E). These results indicated that vacuolar degradation of Fox3p was specific and occurred at both a faster rate and a higher level than non-specific degradation of cytosol or other organelles.

Morphology of pexophagy

We extended our analysis of peroxisome degradation through immunoelectron microscopy using antiserum to Fox3p. Peroxisomes were induced in YTO, and samples were removed at various times following a shift to SD-N medium. Immunogold detection of Fox3p was carried out as described in Materials and Methods. Virtually all of the Fox3p was within the peroxisomes indicating normal import into the matrix (Fig. 3). In both wild-type and *prb1Δ* strains, peroxisomes are seen in the cytosol immediately after shift to SD-N medium (Fig. 3A,B). In wild-type cells, a decrease in peroxisomes is seen over time in SD-N (compare Fig. 3A to E) and intact peroxisomes are not seen within the vacuole lumen suggesting the organelles are being degraded. In contrast, intact peroxisomes were seen to accumulate within the vacuole in the *prb1Δ* strain following the shift to SD-N (Fig. 3D,F). The *prb1Δ* mutant lacks proteinase B and is defective in the breakdown of intravacuolar vesicles (Takeshige et al., 1992). Numerous membrane-enclosed compartments presumably corresponding to autophagic bodies can also be seen in the vacuole lumen of this strain. In many cases, the peroxisomes inside the vacuole appear to be surrounded by a secondary membrane (note the peroxisomes marked by arrows in Fig. 3D). From this analysis, however, we cannot determine whether this enwrapping membrane is the result of a micro- versus macropexophagic process.

Macroautophagy and Cvt pathway mutants are blocked in degradation of Fox3p and pexophagy

In methylotrophic yeasts, peroxisome degradation occurs by both micro- and macropexophagy (reviewed by Klionsky, 1997). To gain further insight into the mechanism of pexophagy in *S. cerevisiae*, we monitored Fox3p following shift from YTO

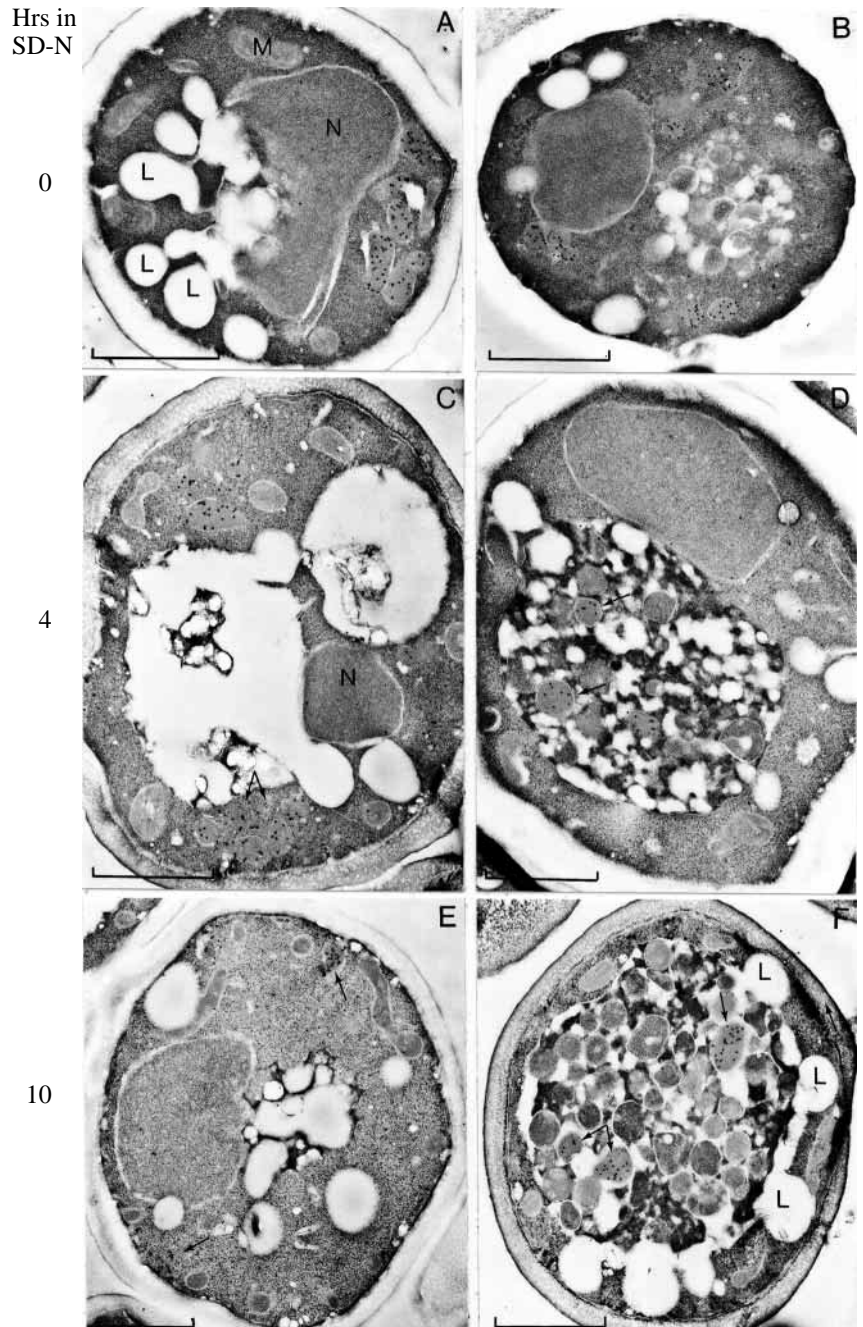


Fig. 3. Peroxisomes accumulate in the vacuole in a *prb1Δ* strain. Wild-type (YW5-1B) and *prb1Δ* (TN122) cells were induced for peroxisomes and chased in SD-N as described in Materials and Methods. Cells were fixed and labeled with Fox3p antiserum and detected with immunogold conjugated secondary antibodies. Representative timepoints are shown as 0, 4, and 10 hours in SD-N chase for wild-type (A,C,E) or *prb1Δ* (B,D,F), respectively. Arrows mark the position of peroxisomes. L, lipid bodies; N, nucleus. Bar, 0.5 μm. Electron micrographs show Fox3p (peroxisome) degradation in wild-type cells and accumulation of peroxisomes in the vacuole in the proteinase B deficient strain (*prb1Δ*).

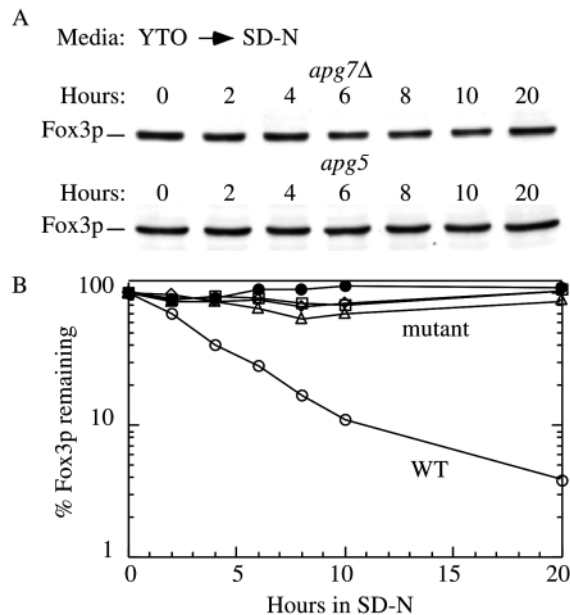


Fig. 4. Mutants in the Cvt and autophagy pathways are defective in their ability to degrade Fox3p. Sample preparation and immunoblots were carried out as described for Fig. 2. (A) Immunoblots of Fox3p in *apg7Δ* and *apg5*. (B) The percentage remaining Fox3p in WT (YW5-1B), *apg1*, *apg8*, *apg10* and *apg14* (open circle, open square, open triangle, open diamond and closed circle, respectively) were quantified as in Fig. 2. *cvt* and *apg* mutations block Fox3p (peroxisome) degradation.

to SD-N in mutants defective in macroautophagy, and the Cvt pathway used for the import of aminopeptidase I (Harding et al., 1995; Tsukada and Ohsumi, 1993). The *apg7/cvt2* mutant was isolated in independent screens by our laboratory and that of Y. Ohsumi. The *APG7* gene was recently cloned and shown to encode a protein with homology to the ubiquitin-E1-like enzyme family (Mizushima et al., 1998). Apg7p is involved in a unique conjugation reaction that covalently links Apg12p to another protein in the autophagy and Cvt pathways, Apg5p. Apg7p and Apg5p are both required for macroautophagy and API import (Mizushima et al., 1998; Kim et al., 1999; Tanida et al., 1999). The *apg7Δ* and *apg5* mutants were blocked in degradation of Fox3p (Figs 4, 6B).

The functions of most of the Apg and Cvt proteins have not been determined. However, some of the proteins are known to interact based on genetic and/or biochemical data, and can be placed into various subgroups. For example, Apg10p is required for formation of the Apg12p-Apg5p conjugate (Mizushima et al., 1998), suggesting that it acts after the E1-homologue Apg7p. The *APG1* gene suppresses the *apg13* mutation suggesting an interaction between these proteins (Funakoshi et al., 1997). Similarly, the *AUT7/APG8* gene suppresses the *aut2* mutation, and the Aut7/Apg8 and Aut2 proteins have been shown to interact through affinity chromatography (Lang et al., 1998). Finally, the Apg6 and Apg14 proteins can be co-immunoprecipitated suggesting that they form a stable complex (Kametaka et al., 1998). We extended our analysis of the effect of autophagy mutants on Fox3p degradation by examining representative mutants from these various groups. In all cases, the mutants were unable to

degrade Fox3p (Fig. 4B). These components may be involved in the common steps for autophagosome or pexophagosome formation and delivery to the vacuole, or direct sequestration by the vacuole membrane, depending on whether uptake occurs by a macro- or micropexophagy mechanism, respectively.

Fox3p is localized to the peroxisomes in the *apg/cvt* mutants

Stabilization of Fox3p could have resulted from a peroxisomal import defect in the *apg/cvt* mutant strains. To rule out this possibility, we examined the subcellular fractionation of Fox3p in a representative *apg* mutants and a control strain defective in peroxisome protein import, *pex7Δ* (Marzioch, 1994). Yeast cells were converted to spheroplasts and lysed osmotically as described in Materials and Methods. The lysate was centrifuged at low speed to remove unlysed spheroplasts. The low speed supernatant fraction was subsequently centrifuged at 25,000 rpm and separated into supernatant (cytosol) and pellet (peroxisome-enriched) fractions (Fig. 5). Pex7p is the peroxisomal targeting signal (PTS) receptor for peroxisomal proteins, such as Fox3p, containing the PTS2 signal. In the *pex7Δ* strain, Fox3p is clearly localized to the supernatant fraction due to its defect in peroxisomal import (Fig. 5). In contrast, both wild-type and *apg7* strains showed correct localization of Fox3p to the pellet fraction suggesting normal import into the peroxisome. Analysis of the cytosolic marker PGK indicated efficient spheroplast lysis, ruling out the possibility that Fox3p in the pellet fraction resulted from intact spheroplasts.

Additional evidence that Fox3p is localized within the peroxisomes of *apg/cvt* mutants is shown by immunoelectron microscopy (Fig. 6). Wild-type and *apg7Δ* cells were grown in YTO to induce peroxisomes. The wild-type cells were examined prior to the induction of pexophagy to prevent the loss of peroxisomes. The *apg7Δ* strain was shifted to SD-N for 10 hours prior to sample preparation. In the *apg7Δ* strain, Fox3p was detected within the peroxisomes in agreement with the results obtained by subcellular fractionation. After 10 hours in SD-N medium, Fox3p was still detected in peroxisomes located in the cytosol in the *apg7Δ* strain.

DISCUSSION

Pexophagy has been studied primarily in the methylotrophic yeasts *H. polymorpha* and *P. pastoris*, however, regulated

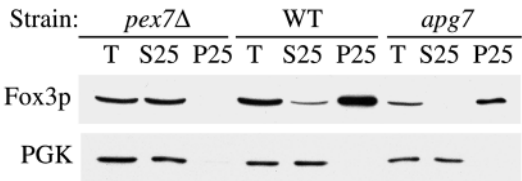


Fig. 5. Fox3p localizes to a peroxisome-enriched fraction in the *apg7* mutant. Subcellular fractionation of *pex7Δ*, wild-type (YW5-1B), and *apg7* was performed on osmotically lysed spheroplasts after induction on YTO as described in Materials and Methods. Immunodetection of Fox3p reveals that it is correctly localized to the 25,000 rpm peroxisome pellet fraction for wild-type and *apg7* strains while the *pex7Δ* strain mislocalizes Fox3p to the supernatant fraction. Detection of the cytosolic marker, PGK, indicates efficient lysis of spheroplasts.

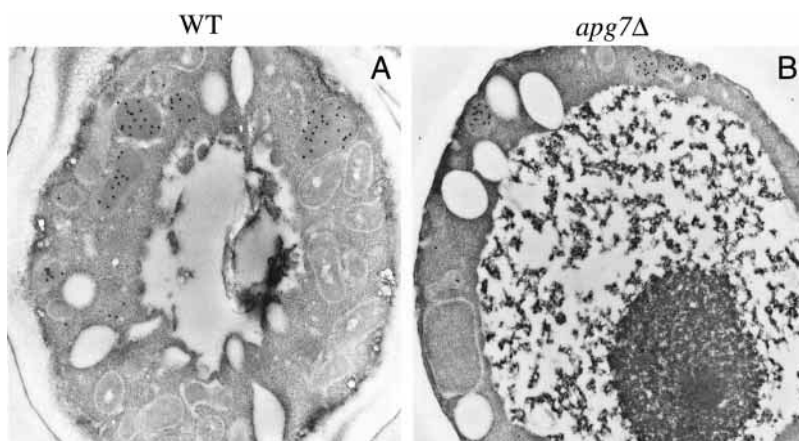


Fig. 6. Fox3p is located within the peroxisomes of the *apg7Δ* strain. Wild-type (MMY011) and *apg7Δ* (MHY1) strains were examined by immunoelectron microscopy as described in Materials and Methods after induction of peroxisomes (A) or following a 10 hour shift to SD-N (B). Fox3p is localized to the peroxisome and is maintained in the cytosol in the *apg7Δ* strain after shifting to conditions that induce pexophagy.

peroxisome degradation has also been demonstrated in *S. cerevisiae* (Evers et al., 1991; Chiang et al., 1996). Despite the genetic advantages of *S. cerevisiae* relative to less tractable yeasts, there are no published studies using this system to generate mutants in the peroxisome degradation pathway. Although pexophagy mutants have been isolated in both *H. polymorpha* and *P. pastoris*, only four genes involved in this process have been characterized. Extensive morphological analyses in *H. polymorpha* and *P. pastoris* have shown that both yeasts utilize a process of macropexophagy where peroxisomes are sequestered within cytosolic vesicles that subsequently fuse with the vacuole. This process is topologically similar to macroautophagy and import of API through the Cvt pathway. We analyzed peroxisome degradation by following the peroxisomal matrix protein Fox3p in *cvt/apg* mutants to determine the extent of overlap among these pathways.

We found that wild-type cells degraded Fox3p when shifted from oleic acid to rich medium, or to nitrogen starvation conditions (Fig. 2). Loss of antigenically detectable Fox3p occurred with a half-time of 3.2 to 5.2 hours, which is slower than the previously reported half-time of 1.5 to ~2.5 hours (Evers et al., 1991; Chiang et al., 1996). This variation may reflect strain differences or effects due to culture conditions. The degradation of Fox3p was vacuole-dependent and the protein was stabilized in a *pep4Δ* (Fig. 2) and *prb1Δ* (Fig. 3) mutant. Intact peroxisomes could be seen to accumulate within the vacuole in strains deficient in vacuolar hydrolases (Fig. 3).

Utilizing various marker proteins, we demonstrated that vacuolar degradation of Fox3p was a specific process. Pho8Δ60p is taken into the vacuole non-specifically by autophagy. Autophagic uptake of cytosol occurs at a basal level in vegetative conditions and is maximally induced to approximately 4% per hour during starvation (Teichert et al., 1989). Vacuolar uptake of Pho8Δ60p is undetectable in rich medium (Scott et al., 1996). In nitrogen limiting conditions, uptake of Pho8Δ60p plateaus at approximately 30% (Noda et al., 1995; Scott et al., 1996). In contrast, Fox3p was extensively degraded (>90%) with similar kinetics in either condition indicating that its loss was not due to non-specific autophagy. Similarly, other organelle markers were not degraded at a rate equivalent to that seen for Fox3p (Fig. 2). While we cannot detect Fox3p after extended incubation in the presence of

glucose, it is likely that a low level of peroxisomes are still present (Veenhuis et al., 1987).

Degradation of Fox3p was blocked in *cvt/apg* mutants that are defective in API import/autophagy (Figs 4, 6). Both subcellular fractionation and immunoelectron microscopy indicated that Fox3p was present within the peroxisomes in the mutant strains, ruling out the possibility that stabilization occurred through cytosolic mislocalization (Figs 5, 6). The overlap between pexophagy and the Cvt/Apg pathways allows us to present a model for peroxisome degradation in *S. cerevisiae* (Fig. 7). Peroxisomes are induced when yeast are grown on oleic acid as the sole carbon source. Upon detection of preferable carbon sources (such as glucose) which completely repress synthesis of essential peroxisomal proteins, the cell no longer needs to maintain peroxisomes in such a proliferated state and pexophagy is induced. If uptake occurs by a macroautophagic mechanism (Fig. 7A), a double membrane structure, the pexophagosome, forms around peroxisomes and encapsulates them for delivery to the vacuole. Subsequently, the outer membrane of the pexophagosome fuses with the vacuole and releases the peroxisome encapsulated within the remaining inner membrane of the pexophagosome. Resident vacuolar hydrolases break down the membrane and peroxisome into their macromolecular

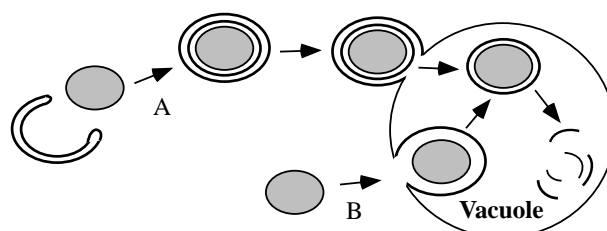


Fig. 7. *S. cerevisiae* induces peroxisomes (stippled) when grown on oleic acid as the sole carbon source. Upon addition of glucose and/or shift to nitrogen starvation, peroxisomes are degraded by macro- or micropexophagy. (A) In macropexophagy, peroxisomes are sequestered within cytosolic vesicles that subsequently fuse with the vacuole. (B) Micropexophagy involves engulfment of peroxisomes at the vacuole surface. Both mechanisms generate an intravacuolar vesicle that is broken down by vacuolar hydrolases. The machinery involved in peroxisome degradation overlaps with that of the Cvt and autophagy pathways.

components. Alternatively, peroxisomes are engulfed directly at the vacuole surface by a micropexophagic mechanism (Fig. 7B). Breakdown occurs similar to the macropexophagic model, following scission of the sequestering membrane from the vacuolar membrane. Our present data do not allow us to distinguish between these two possibilities.

The observation that Fox3p degradation is more rapid and specific than can be accounted for by autophagy implies a mechanism for tagging and detecting peroxisomes. Molecular components that are specific to pexophagy have not yet been identified. Some mutants such as *cvt9* are specific for the Cvt pathway and do not block autophagy. Inhibition of Fox3p degradation in these mutants (data not shown) provides a connection between the specific cytoplasm to vacuole targeting pathway and the specific aspects of pexophagy.

Recently, the *P. pastoris* *GSA7* gene was cloned and shown to be homologous to *APG7* of *S. cerevisiae* (Yuan et al., 1999). The two genes can functionally substitute for one another in API import or pexophagy, respectively (Yuan et al., 1999; Kim et al., 1999). This finding coupled with the data in the present study suggest that a large number of the *GSA*, *PAG*, and *PDD* genes may be homologous to *APG*, *AUT* and *CVT* genes. In agreement with this prediction, a *Pichia pastoris* homologue to *CVT9*, *GSA9*, has been identified and shown to be required for peroxisome degradation (P. Stromhaug and W.A. Dunn, personal communication). We continue our search for additional components involved in pexophagy in *S. cerevisiae* to better understand how nutrient stimulated signals are sensed and transduced into complex mechanisms of membrane dynamics.

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